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The Fas Death Factor

Shigekazu Nagata* and Pierre Golstein

Fas ligand (FasL), a cell surface molecule belonging to the tumor necrosis factor family, binds to its receptor Fas, thus inducing apoptosis of Fas-bearing cells. Various cells express Fas, whereas FasL is expressed predominantly in activated T cells. In the immune system, Fas and FasL are involved in down-regulation of immune reactions as well as in T cell–mediated cytotoxicity. Malfunction of the Fas system causes lymphoproliferative disorders and accelerates autoimmune diseases, whereas its exacerbation may cause tissue destruction.

Homeostasis of multicellular organisms is controlled not only by the proliferation and differentiation of cells but also by cell death (*1*). The death of cells during embryogenesis, metamorphosis, endocrine-dependent tissue atrophy, and normal tissue turnover is called programmed cell death. Most of programmed cell death proceeds by apoptosis, a process that includes condensation and segmentation of nuclei, condensation and fragmentation of the cytoplasm, and often extensive fragmentation of chromosomal DNA into nucleosome units.

Apoptosis in vertebrate development often occurs by default when cells fail to receive the extracellular survival signals needed to suppress an intrinsic cell suicide program (*2*); the survival factors can be produced by neighboring cells of a different type (a paracrine mechanism), or of the same type (an autocrine mechanism). In contrast, in the immune system there are situations where cells actively kill other cells; for example, cytotoxic T lymphocytes (CTLs) or natural killer (NK) cells induce apoptosis in their targets such as virus-infected cells or tumor cells (*3*). In these cases, an effector molecule expressed at the surface of CTLs or NK cells or a soluble cytokine produced by these effector cells is thought to be responsible for target cell death.

Molecular and cellular characterization of Fas, a cell surface protein recognized by cytotoxic monoclonal antibodies, revealed its role as a receptor for a Fas ligand (FasL) (*4*). When FasL binds to Fas, the target cell undergoes apoptosis. Spontaneous mutations for Fas and FasL have been identified in mice, and from the phenotypes of these mutants and from studies on mechanisms of cytotoxicity, it was concluded that the Fas-FasL system is involved not only in CTL-mediated cytotoxicity but also is down-regulation of immune responses. In this article, we summarize current knowledge on Fas and FasL and discuss their physiological and pathological roles in the immune system.

Fas and Fas Ligand

In 1989 two groups independently isolated mouse-derived antibodies that were cytolytic for various human cell lines (*5*, *6*). The cell surface proteins recognized by the antibodies were designated Fas and APO-1, respectively. The antibody to Fas (anti-Fas) was an immunoglobulin M (IgM) antibody, whereas the antibody to APO-1 was classified as IgG3. The Fas complementary DNA

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(cDNA) was isolated by expression cloning from a cDNA library of human KT-3 lymphoma cells which abundantly express Fas (7). Human Fas consists of 325 amino acids with a signal sequence at the NH_2-terminus and a membrane-spanning region in the middle of the molecule, indicating that Fas is a type I membrane protein.

The function of Fas was assessed by the establishment of mouse cell transformants that constitutively expressed human Fas. When the transformed cells were treated with the antibody to human Fas, the cells died by apoptosis within 5 hours, which indicated that Fas can transduce an apoptotic signal and that anti-Fas works as an agonist. The subsequent purification of human APO-1 antigen and molecular cloning of its cDNA established the identity of APO-1 and Fas (8).

The structure of Fas indicated that it belongs to the tumor necrosis factor (TNF) and nerve growth factor (NGF) receptor family (Fig. 1A) (7–9). This family includes two TNF receptors (TNF-R1 and TNF-R2), the low-affinity NGF receptor, CD40, OX40, CD27, 4-1BB, and CD30 (10). The extracellular regions of members of this family consist of three to six cysteine-rich domains. The amino acid sequence of the extracellular region is relatively conserved, whereas the cytoplasmic region is not, except for some similarity between Fas and TNF-R1 (7, 8). Subsequent mutational analyses of Fas and TNF-R1 indicated that the cytoplasmic domain (about 70 amino acids) conserved between Fas and TNF-R1 is necessary and sufficient for transduction of the apoptotic signal (11). This domain was therefore designated a death domain.

In humans the single Fas gene per haploid genome is located on the long arm of chromosome 10 (12), whereas in the mouse, the gene is on chromosome 19 (9). The human and mouse genes span 12 kb and more than 70 kb, respectively, and both genes comprise nine exons (13). As for the expression patterns of Fas in various tissues and cell lines, there is considerable variability. Many tissues and cell lines weakly express Fas, but abundant expression was found in mouse thymus, liver, heart, lung, kidney, and ovary (9). Unlike mouse thymocytes human thymocytes only weakly express Fas. In mouse thymocytes, Fas is expressed in almost all populations except for double-negative ($CD4^-CD8^-$) thymocytes (14). Fas is highly expressed in activated mature lymphocytes (5) or lymphocytes transformed with human T cell leukemia virus (HTLV-I), human immunodeficiency virus (HIV), or Epstein-Barr virus (EBV) (15, 16). Some other tumor cells also express Fas, although the expression level is low compared with that of lymphoblastoid cells. The expression of Fas is up-regulated by interferon-γ (IFN-γ) in various cell lines (6, 7, 9), or by a combination of IFN-γ and TNF-α in human B cells (17), which may explain the enhancement of the cytotoxic activity of anti-Fas by these cytokines (6). The induction mechanism of Fas expression or the promoter elements of the Fas gene have not been intensively studied.

The structure of Fas suggested that Fas was a receptor for an unknown cytokine. In 1993, Rouvier *et al.* (18) reported on a subline from a cytotoxic T cell hybridoma (PC60) between a mouse CTL cell line and a rat lymphoma. The subline (PC60-d10S, d10S for short) could kill target cells expressing Fas but not target cells which did not express Fas. Soluble forms of Fas (Fas-Fc) and TNF receptor (TNFR-Fc) were constructed by fusing their extracellular regions to human IgG (19). The Fas-Fc but not TNFR-Fc inhibited the CTL activity of d10S cells, indicating that the d10S cells expressed a FasL and that this FasL played a major role in d10S-mediated cytotoxicity. The FasL on d10S cells could be stained by biotinylated Fas-Fc, and a subline of the d10S cells (d10S16), which expressed ~100 times more FasL than did the original d10S cells, was then established by repeated fluorescence-activated cell sorting. The d10S16 cells showed about 100 times more CTL activity against the Fas-expressing cells than did the d10S cells.

FasL was purified to homogeneity from the solubilized membrane fraction of d10S16 cells by means of affinity-chromatography with Fas-Fc (19). The purified FasL was a protein with a molecular weight ratio of ~40,000, and it showed strong cytotoxic activity against Fas-expressing cells. Rat FasL cDNA was then cloned by expression cloning from the d10S subline with Fas-Fc (20), and mouse and human FasL cDNAs were subsequently cloned by cross-hybridization (21, 22). FasL has no signal sequence at the NH_2-terminus, but it has a domain of hydrophobic amino acids in the middle of the molecule, indicating that it is a type II membrane protein with the COOH-terminal region outside the cell. Mouse and human

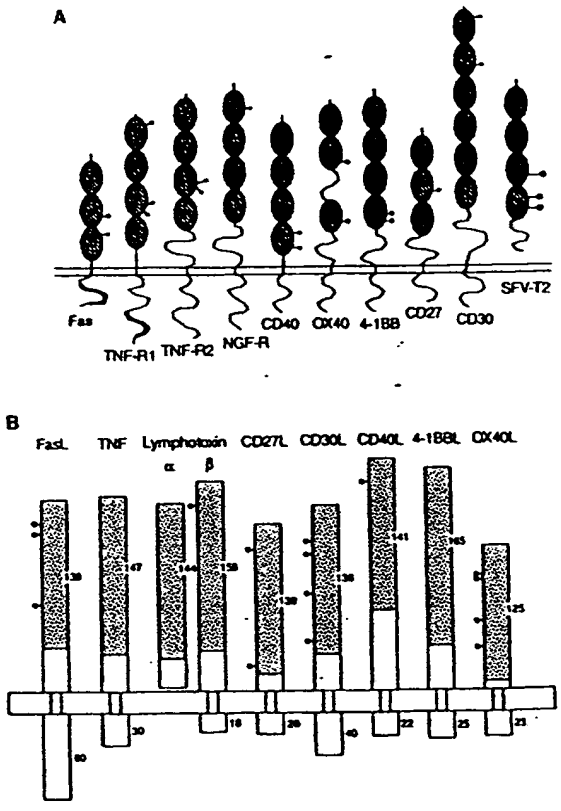

Fig. 1. TNF and its receptor family. (A) The TNF and NGF receptor family. Members of the TNF-NGF receptor family are schematically shown. These include Fas, TNF-R1, TNF-R2, NGF receptor, B cell antigen CD40, T cell antigens OX40, 4-1BB, and CD27, Hodgkin's lymphoma antigen CD30, and the soluble protein coded by Shope fibroma virus (SFV-T2). The striped regions represent cysteine-rich subdomains; each member of the family contains three to six of them. The death domains (about 80 amino acids) in the cytoplasmic regions of Fas and TNF-RI, which have some similarity, are shown as bold lines. The symbol —● indicates an N-glycosylation site. [Reproduced from (4), S. Nagata, with permission from Academic Press.] (B) The TNF family. Members of the TNF family are schematically shown. These include Fas ligand, TNF, the α and β subunits of lymphotoxin, CD27 ligand, CD30 ligand, CD40 ligand, 4-1BB ligand, and OX40 ligand. These members are type II membrane proteins, except for the α subunit of lymphotoxin which is a secretory protein. The shaded portion of each member is the extracellular region which shows significant similarity (25 to 30% identity) among the members. The number of the amino acids in the homologous region and the cytoplasmic region are indicated. The symbol —● indicates an N-glycosylation site.

FasLs are 76.9% identical at the amino acid sequence level, and they are functionally interchangeable. A stretch of about 150 amino acids in the extracellular region of the FasL has significant homology to the corresponding region of other members of the TNF family which includes TNF, lymphotoxin (LT), CD40 ligand, CD27 ligand, CD30 ligand, and OX40 ligand (Fig. 1B). Expression of recombinant FasL on the cell surface of fibroblast-like COS cells was sufficient to induce apoptosis in Fas-expressing target cells within a few hours (*20*), indicating that FasL is a death factor and Fas is its receptor (*4*).

A single FasL gene is located on human and mouse chromosome 1 (*21, 22*) in the neighborhood of the OX40 ligand gene, another member of the TNF family (*23*). The FasL gene comprises five exons, and its organization is similar to those of TNF-α and LT-β. Among the various cell lines examined, only activated T cell lines expressed FasL (*24*). FasL messenger RNA (mRNA) was not detected in B cells or macrophage, fibroblast, endothelial, or thymic stroma cell lines. The exception was the testis, where abundant FasL expression was found in mouse and rat (*20*), but interestingly, not in human (*25*). The expression of FasL in T cells could be rapidly induced by activation with phorbol 12-myristate 13-acetate (PMA) and ionomycin or T cell receptor engagement (*24*). Herbimycin A and genistein (protein tyrosine kinase inhibitors) as well as cyclosporin A (an inhibitor of the calmodulin-dependent protein phosphatase calcineurin) inhibited the induction of FasL expression. It is likely that some tyrosine kinases and calcineurin are involved in the activation of the FasL gene through the T cell receptor, as has been found for the induction of other lymphokine genes. The nuclear factor–κB (NF-κB) or interferon regulatory factor–1 (IRF-1) element found in the promoter region of the FasL gene may be responsible for this induction (*21*).

A Death Signal from Fas

Signaling by means of Fas leads to apoptotic cell death, with characteristic cytoplasmic and nuclear condensation and DNA fragmentation (*5, 7, 20, 26, 27*). Triggering this pathway requires the cross-linking of Fas either with antibodies to Fas (*28*), with cells expressing FasL, or with purified FasL (*19*). Similar to TNF, the soluble form of FasL has a trimeric structure in solution (*29*). Therefore, it is likely that the cross-linking of Fas molecules, rather than just their engagement, leads to further signaling within the cell.

Cell death via Fas does not require the presence of a nucleus or DNA fragmentation. Cells enucleated with cytochalasin B undergo apoptosis (that is, the characteristic cytoplasmic lesions appear) when triggered by the Fas-based mechanism with anti-Fas or with cytotoxic T cells (*27*). This is in line with the fact that a nucleus is not required in other apoptotic death systems, such as staurosporin-induced apoptosis (*30*). These results, as well as others showing the death-inducing ability of cytoplasmic extracts on isolated nuclei (*31*), indicate that "nuclear death" is only secondary to essential cytoplasmic death events. What are these cytoplasmic death events and how are they related to the first irreversible step of cell death? The answers are not better known in the case of death via Fas than in the case of death induced by other means. Answers to these questions may provide a biochemical definition of cell death and be of considerable practical importance.

Additional results showed that the Fas-triggered pathway to death is independent of extracellular Ca^{2+} (*18, 32*) and does not require macromolecular synthesis (*6, 7, 33–35*). As for many other death pathways, the cellular background plays an essential role in the interpretation and modulation of the Fas-originating signal. Thus, cell sensitivity or resistance involves factors other than just the level of expression of Fas (*26, 33, 36, 37*). The molecular basis underlying this variability in response may depend on which intracellular molecules are available to bind given segments of the Fas intracytoplasmic region, or depend on other molecules such as Bcl-2 and related proteins that modulate cell death (*38*). In fact, the Fas-transduced cell death is partially inhibited by overexpression of Bcl-2 (*39*), but it is completely inhibited by coexpression of

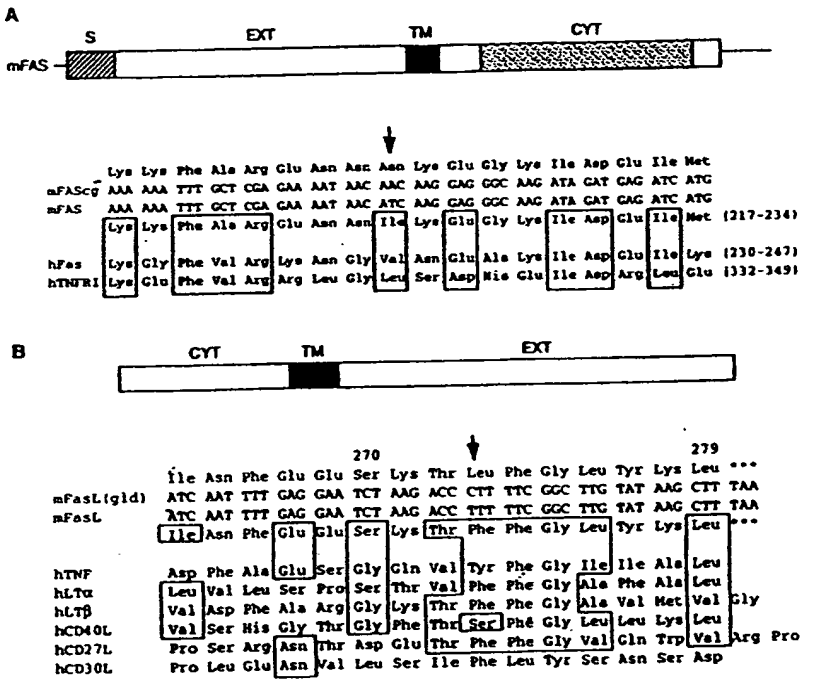

Fig. 2. Mutations in Fas and FasL in *lpr* and *gld* mice. (A) A point mutation in the cytoplasmic region of the Fas gene of lpr^{cg} mice. The upper panel shows the structure of Fas. S, signal sequence; EXT, extracellular region; TM, transmembrane region; CYT, cytoplasmic region. The Fas death domain, which has similarity with that of TNF-R1, is shown as the shaded area. The lower panel shows the nucleotide sequence and the predicted amino acid sequence of the wild-type (mFAS) and the mutant Fas (mFAScg). The amino acid sequence of the corresponding region of human Fas (hFas) and TNF-RI (hTNFRI) are also shown. Sets of three identical or homologous amino acid residues at one aligned position are boxed. The arrow indicates the position of the Fas mutation in *lpr* mice. [Reproduced from (*57*) with permission from the author and *Nature*, copyright Macmillan Magazines.] (B) A point mutation in the extracellular region of FasL of *gld* mice. The upper panel shows the structure of FasL. The lower panel shows the nucleotide and amino acid sequences of the mutant FasL [mFasL(gld)] and wild type (mFasL). The arrow indicates the position of the mutation in FasL of *gld* mice. Amino acid sequences of the corresponding region of the other members of the TNF family (TNF, LT-α, LT-β, CD40L, CD27L, and CD30L) are also shown. The amino acids of favored substitutions in more than four members are boxed. [Reproduced from (*22*), T. Takahashi *et al.*, with permission from the author and *Cell*, copyright Cell Press.]

Bcl-2 and its binding protein BAG-1 (40). It is not yet known whether molecules such as interleukin-1 converting enzyme (ICE) protease and Cdc2 kinase, which have been implicated in some cell death systems (41), play a role in Fas-induced cell death.

Because Fas and TNF-R1 have homologous cytoplasmic death domains, one might expect that death transduced by means of one or the other of these surface receptors would have similar characteristics. This may not be the case. Unlike TNF-R1–transduced death in the same type of cells, Fas-transduced death is not blocked by manganese superoxide dismutase (MnSOD), metallothionein, plasminogen activator type 2, A20, or mitochondrial inhibitors (37, 42), and it does not activate the transcription factor NF-κB. The latter observation in particular is not completely consistent with indications that it is the death domain of TNF-R1, homologous to that of Fas, which triggers the acidic sphingomyelinase that leads to activation of NF-κB (43). Moreover, the Fas-induced cell death is quicker than that induced by TNF-R1 (44).

Why do these similar receptors transduce death pathways with such different characteristic? One possibility, still unsubstantiated, is that other parts of the cytoplasmic domains of these receptors generate other signals that modulate the main cell death signal. Indeed, distinct signals can originate from distinct parts of the TNF-R1 cytoplasmic region (43). Recently, cytosolic molecules have been identified that can associate with TNF-R2 and CD40, which are other members of the TNF-NGF receptor family (45). It would be interesting to examine whether these cytosolic molecules or related molecules can associate with Fas or TNF-R1.

A fast-developing field of research deals with the possibility that Fas signals by means of the complex lipid ceramide. Ceramide is one of the products that results from the breakdown by sphingomyelinases of sphingomyelin, a sphingosine–fatty acid–phosphorylcholine molecule found in the plasma membrane and cytoplasm. The involvement of ceramide in the Fas death pathway has been suggested by the following experiments. When various Fas-bearing cells were incubated with anti-Fas for 5 min, acidic sphingomyelinase activity was detected, there was partial hydrolysis of sphingomyelin, and the amount of ceramide increased. Cell-permeable synthetic C2-ceramide itself, when added to the culture medium, was able to induce apoptosis in less than 3 hours (46). The addition of sphingomyelinase or ceramide extracellularly led to the induction of cell death with DNA fragmentation and apoptotic morphology (47). Downstream signaling by ceramide might take place by means of a ceramide-activated protein kinase (48), which could account in part for the phosphorylations observed within 1 min after antibody-mediated Fas ligation on Jurkat cells; protein kinase inhibitors block the resulting DNA fragmentation and cell death (49).

The *lpr* and *gld* Mutations

The mouse spontaneous mutants *lpr* (lymphoproliferation) and *gld* (generalized lymphoproliferative disease) carry autosomal recessive mutations (50) on mouse chromosome 19 and 1, respectively (51). MRL *lpr/lpr* and MRL *gld/gld* mice develop lymphadenopathy and splenomegaly and produce large quantities of IgG and IgM antibodies including anti-DNA and rheumatoid factor (52). They develop nephritis or arthritis and die at around 5 months of age. The allelic mutation (lpr^{cg}) in the *lpr* locus (53), distinct from the original *lpr* mutant, causes a weak *lpr* phenotype in double heterozygotes with *gld* ($lpr^{cg}/+$, $gld/+$), in addition to the *lpr* phenotype of its homozygous mutation. The other strains of mice carrying *lpr* or *gld* mutations develop lymphadenopathy and splenomegaly, but not nephritis or arthritis (54). Because the wild-type MRL mice develop weak and delayed autoimmune disease, it is likely that *lpr* and *gld* mutations accelerate or worsen the autoimmune disease rather than induce it.

The lymphocytes that accumulate in the lymph nodes and spleen in *lpr* or *gld* mice are arrested at the G_0-G_1 stage of the cell cycle and express the T cell marker Thy-1 and the B cell marker B-220 (52). These cells express a rearranged T cell receptor but not a rearranged IgG gene. Because neonatal thymectomy prevents the accumulation of lymphocytes, it seems that it is T cells that accumulate in the mutants. However, the CD4 and CD8 antigens, which are usually expressed on mature T cells, are not expressed on the lymphocytes that accumulate in *lpr* or *gld* mice. The CD4 and CD8 gene loci are demethylated in these lymphocytes, and the administration of anti–Thy-1, anti-CD4, or anti-CD8 inhibits development of the lymphadenopathy (52). When mature $CD4^+$ T cells from *lpr* mice were transplanted into wild-type mice, these T cells became double-negative T cells (55), suggesting that the cells accumulating in *lpr* mice are derived from mature, single-positive ($CD4^+CD8^-$ or $CD4^-CD8^+$) T cells.

Although *lpr* and *gld* mutations are nonallelic mutations, they show a similar phenotype. In 1991, Allen *et al.* (56) carried out a series of bone marrow transplantation experiments among *lpr*, *gld*, and wild-type mice. From these experiments they concluded that *lpr* and *gld* are mutations in genes encoding a pair of interacting proteins: *gld* may affect a soluble or membrane-bound cytokine, whereas *lpr* may affect its receptor. The Fas gene was mapped to a location near the *lpr* locus on mouse chromosome 19 (9). Northern (RNA) hybridization analysis indicated that, in contrast to wild-type mice, *lpr* mice express very little Fas mRNA in the liver and thymus (57). Characterization of the Fas gene in *lpr* mice indicated that an early transposable element (ETn), a variety of mouse endogenous retrovirus, is inserted into intron 2 (58). The ETn carries a polyadenylate signal (AATAAA) on the long terminal repeat (LTR) sequence, which causes premature termination of the Fas RNA transcript.

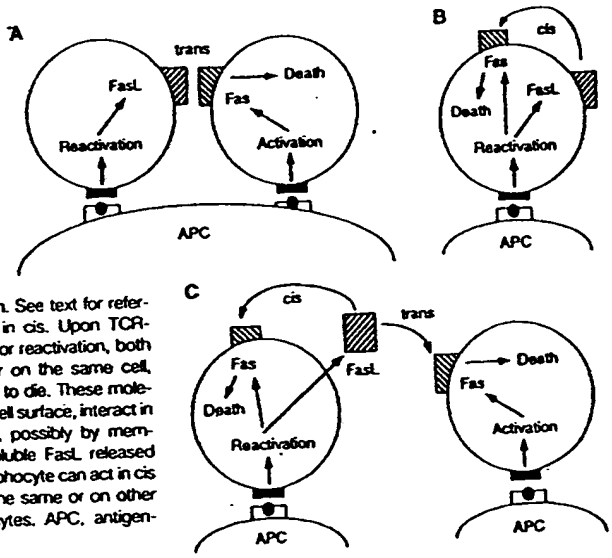

Fig. 3. A schematic representation of three mechanisms that may be involved in Fas-based immune down-regulation at the lymphocyte level. (A) Fas and its ligand, FasL, may be expressed on different cells, leading to Fas-based death in trans through a cytotoxicity-like mechanism. See text for references. (B) "Suicide" in cis. Upon TCR-transduced activation or reactivation, both Fas and FasL appear on the same cell, which is then induced to die. These molecules, situated at the cell surface, interact in an unknown manner, possibly by membrane folding. (C) Soluble FasL released from an activated lymphocyte can act in cis or trans, that is, on the same or on other Fas-bearing lymphocytes. APC, antigen-presenting cell.

Small mRNAs coding for exons 1 and 2 were found in the thymus and liver of *lpr* mice. When the ETn in the Fas gene of *lpr* mice was inserted into the corresponding intron in a mammalian expression vector, the expression efficiency of the vector was reduced to a few percent of that of the original vector.

These results demonstrated that transcription of the Fas gene is impaired in *lpr* mice by an insertion of a transposable element in an intron of the gene. However, this mutation is leaky. Intact Fas mRNA could be found in the thymus and liver of the *lpr* mice at the level of several percent that of the wild-type mouse. The lpr^{cg} mice express normal size Fas mRNA at a level similar to that of wild-type mice (57). However, the mRNA carries a T→A point mutation in the Fas cytoplasmic region (Fig. 2A). This mutation changes an isoleucine to an asparagine in the death domain, abolishing the ability of Fas to transduce the apoptotic signal. Together, these results indicate that *lpr* mutations are loss-of-function mutations of the Fas gene. Recent results with transgenic mice, in which the expression of the Fas gene in T cells of *lpr* mice corrected the phenotype (59), confirm this conclusion.

The FasL gene was similarly mapped on mouse chromosome 1 where the *gld* mutation is localized (22). There is no rearrangement of the FasL gene in the *gld* mice, but there is a point mutation near the COOH-terminus of the coding region (Fig. 2B). This mutation changes a phenylalanine to a leucine in the extracellular region and abolishes the ability of FasL to bind to Fas.

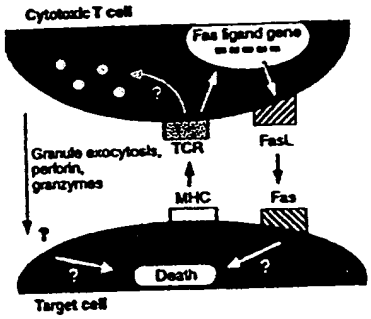

Fig. 4. A schematic representation of two major pathways of T cell–mediated cytotoxicity. The sensitizing antigen, presented by the MHC at the target cell surface, is recognized by the T cell receptor (TCR) at the effector cell surface. The engaged TCR-CD3 complex then transduces within the effector cell a signal toward the granule exocytosis mechanism, and a signal toward the effector cell nucleus that leads to transcription of, in particular, the FasL gene. Swift expression of the FasL protein then leads to engagement of Fas at the target cell surface, signaling target cell death. Interactions of molecules other than TCR-MHC and the Fas-FasL pairs may further modulate the outcome of the effector–target cell encounter. The question marks indicate some of the steps in these processes for which information is particularly incomplete. TCR engagement leads to increased amounts of FasL mRNA, assumed here to be due to increased transcription, although an increase in message stability is not ruled out.

Fas-Mediated Death of Lymphocytes

As described above, the mouse spontaneous mutations *lpr* and *gld* are loss-of-function mutations of Fas and FasL, respectively. The abnormal accumulation of lymphocytes in *lpr* and *gld* mice suggested that Fas and FasL may be involved in normal lymphocyte death. In the life of lymphocytes, both T cells and B cells normally die at various stages of their development. Precursor T cells originate in the bone marrow and migrate into the thymus, where they mature into single-positive ($CD4^+CD8^-$ or $CD4^-CD8^+$) T lymphocytes. The T cells that can interact with self–major histocompatibility complex (MHC) expressed in the thymus are positively selected (positive selection), whereas those that cannot die by apoptosis. On the other hand, T cells that strongly react with self antigen complexed with self-MHC are induced to undergo apoptosis (negative selection) (60). More than 95% of the T cells that immigrate into the thymus die there; the remaining 5% migrate to the peripheral lymphoid organs as mature T lymphocytes (61). In the periphery, the mature T cells again undergo an additional selection process. Those that interacted with the self antigens expressed only in peripheral tissues would die (peripheral clonal deletion) (62). Furthermore, there must be some mechanism in the periphery to eliminate lymphocytes after they have been activated by antigen to ensure that the organism does not fill up with activated lymphocytes (63).

The positive and negative selections in the thymus are apparently normal in *lpr* mice (64), indicating that a Fas-mediated mechanism is unlikely to be involved. On the other hand, several groups showed that peripheral clonal deletion and the elimination of activated T cells are impaired in *lpr* and *gld* mice (65, 66). Apparently, antigenic stimulation first triggers the proliferation of mature T cells, which are later eliminated by apoptosis. In *lpr* or *gld* mice, an antigen can stimulate the proliferation of mature T cells, but the subsequent death process is severely retarded, both in vitro and in vivo. These results indicate that the Fas system is normally involved in both the clonal deletion of autoreactive T cells in peripheral lymphoid organs and the elimination of activated T cells after they have responded to foreign antigens.

As described below, the Fas system is one of the mechanisms that cytotoxic T cells use to kill infected target cells. A similar cytotoxic mechanism may operate to delete activated T cells. Although mature T cells constitutively express Fas, activation by antigens up-regulates this expression and makes the T cells sensitive to Fas-mediated apoptosis (26, 33). At the same time, activation by antigens induces FasL expression on cytotoxic T cells (24, 67), which are now able to kill Fas-expressing activated lymphocytes by a Fas-based mechanism. The Fas and FasL may interact on the same cell (66) (Fig. 3B) or on different cells (67) (Fig. 3A). In addition, FasL may be released from the activated cytotoxic cells and then activate Fas from solution (29, 68) (Fig. 3C). In each case, the cytotoxicity is not directed against nonself or modified self, but against activated self.

In the absence of a properly functioning Fas-FasL system, such as in *lpr* or *gld* mutants, activated lymphocytes accumulate (69), and because these cells are not efficiently eliminated, autoimmune disease is enhanced. However, other mechanisms may also contribute to the elimination of activated lymphocytes, and there is evidence that these other mechanisms may predominate in young mice, whereas the Fas-based mechanism may be essential in older mice (70, 71).

B cells are also thought to die by apoptosis at several steps of their development (72). During development in the bone marrow, the B cells that are strongly reactive to self components are deleted, apparently by a Fas-independent mechanism (71). The surviving B cells then migrate to peripheral lymphoid organs where they can be activated by antigen. As with T cells, Fas may be involved in the deletion of B cells activated by the self or foreign antigens in the periphery. Activation of mature B cells causes the expression of Fas (5) and renders the cells sensitive to Fas-mediated killing by anti-Fas (73). It is possible that the FasL on activated T cells binds to Fas on activated autoaggressive B cells and kills them by apoptosis. This process would be blocked in *lpr* or *gld* mice, and the B cells that escape deletion may be responsible for the production of a large quantity of immunoglobulins, including autoantibodies, in these mutant mice.

Fas-Based T Cell–Mediated Cytotoxicity

Cytotoxic T lymphocytes (CTLs) are the main effector arm of the immune system responsible for eliminating virus-infected cells. The CTLs can both specifically recognize and lyse their targets. What mechanism or mechanisms are involved in lysis? A well-

known perforin-granzyme–based mechanism (74) does not account for all examples of CTL killing, so a search has begun for alternative mechanisms.

A clue was provided by the demonstration that a CTL hybridoma subline (d10S) (35), upon activation with phorbol 12-myristate 13-acetate (PMA) and ionomycin, lysed Fas-positive but not Fas-negative cells (18). Additional evidence for the existence of Fas-based cytotoxicity came from blocking experiments with the soluble form of Fas (Fas-Fc) or anti-Fas under nonlytic conditions. With these reagents, many T cell lines were found to exert Fas-based cytotoxicity (32, 34, 75, 76).

It has long been known that part of T cell–mediated cytotoxicity was Ca^{2+}-independent (77), whereas the perforin-granzyme–based mechanism was Ca^{2+}-dependent at several levels (78). The cytotoxicity by preactivated d10S cells was Ca^{2+}-independent (35). The presence of EGTA-Mg^{2+} in cytotoxicity assays suppresses the perforin-based mechanism and allows one to study the Fas-based mechanism (18), even in situations where both mechanisms would normally operate simultaneously. In the presence of EGTA-Mg^{2+}, the antigen-specific cytotoxicity expressed by in vitro–raised and in vivo–raised cytotoxic T cells was shown to be Fas-based (18, 79).

The Fas-based cytotoxicity by CTLs can be divided into two processes. First, on recognition of the target cell by the cytotoxic cell, FasL expression is induced in the cytotoxic cell. Second, engagement of FasL on the cytotoxic cells and Fas on the target cell leads to activation of an intrinsic suicide program in the target cell. Interestingly, transfection of FasL cDNA into fibroblast-like recipient cells is enough to render these cells cytotoxic (20), showing that expression of the FasL may be sufficient to make even a nonlymphoid cell cytotoxic.

Which T cells are able to exert Fas-based cytotoxicity? Among the classical $CD4^+$ T helper (T_H) subpopulations (80), T_H1 cells can express the FasL and lyse in a Fas-based manner much more readily than T_H2 cells (32, 75). Neither helper T cell population usually expresses the perforin-based mechanism of lysis. In contrast, $CD8^+$ cells, which are the professional cytotoxic T cells, usually express both the Fas-based and the perforin-based mechanisms (18, 24, 32, 34).

Thus, two mechanisms of cytotoxicity, perforin-based and Fas-based, have been molecularly defined (Fig. 4). There seems to be little cross-talk between these mechanisms, because cytotoxic cells from perforin knock-out mice (79), as well as cytotoxic cell lines that do not express perforin (81), can lyse by means of the Fas pathway, whereas *gld* cytotoxic cells and *lpr* target cells can lyse or be lysed, respectively, by means of the perforin pathway (18, 67, 79, 82). No other mechanism of cytotoxicity has been found, at least in short-term in vitro cytotoxicity tests (79, 81), suggesting that there are only two main mechanisms of T cell–mediated cytotoxicity, Fas-based and perforin-based mechanisms.

Fas and FasL in Pathology

As described above, the Fas system is involved in eliminating normal activated lymphocytes as well as probably some virus-infected cells. The loss-of-function mutations of Fas or FasL causes activated lymphocytes to accumulate, and in mice this produces autoimmune disease. An injection of antibodies to Fas kills adult mice within a few hours (83). These results suggest that the Fas system may have a role in human pathology in two different ways.

First, diseases may be caused by a malfunction of the Fas system. The *lpr* and *gld* mutations in mice are in this category, and several patients have been described with phenotypes similar to that of *lpr* mice (84). It is possible that these patients have defects in Fas or FasL. Indeed, patients with altered Fas were recently reported (85). The *lpr* and *gld* mice produce large amounts of immunoglobulin, including autoantibodies, and develop an autoimmune disease that resembles human systemic lupus erythematosus (SLE) (52). Cheng *et al.* (86) detected an elevated level of the soluble form of Fas in the serum of some human SLE patients. This soluble form of Fas seemed to be produced by a Fas mRNA that was generated by alternative splicing and that encoded a soluble form of Fas protein lacking a transmembrane domain. Because this form of Fas could induce lymphadenopathy and splenomegaly, Cheng *et al.* (86) suggested that it inhibits Fas-mediated elimination of activated lymphocytes and thereby causes the SLE phenotype. Although this is an interesting possibility, it will be necessary to examine more patients to test this hypothesis.

The *lpr* phenotype is dependent on background genes in the mouse (54). MRL mice carrying the *lpr* mutation develop nephritis or arthritis, whereas C3H mice carrying the *lpr* mutation do not, indicating that genes other than Fas, such as those regulating autoimmunity, are involved in Fas-related pathology (87). It may be noteworthy that nitric oxide (NO) synthase expression and NO production are increased in MRL *lpr/lpr* mice, and spontaneous glomerulonephritis and arthritis can be reduced by orally administered N^G-monomethyl-L-arginine which inhibits NO production (88). Active transforming growth factor–β (TGF-β) is overproduced in *lpr* mice, possibly as a homeostatic mechanism for suppressing exaggerated and inappropriate immunostimulation. This apparently leads to a failure of polymorphonuclear leukocytes to migrate to the site of bacterial infection, diminishing the host defense against bacterial infection and thus increasing the bacterial burden (89). This situation is reminiscent of the increased risk of bacterial infection observed in patients with autoimmune disease.

A second category of Fas-related diseases may be caused by excessive activity of the Fas system. There is growing circumstantial evidence that Fas might be involved in the death of $CD4^+$ T cells during the course of an HIV infection. Fas is abundantly expressed on T lymphocytes of HIV-infected children (15) and in retrovirus-induced immunodeficiency syndrome in mice (90). In unfractionated human peripheral blood lymphocytes (PBLs), cross-linking of CD4 molecules, either by an antibody or by the HIV envelope protein gp160, up-regulated the expression of Fas on the PBLs, which closely correlated with the occurrence of apoptotic cell death (91). Injection of mice with antibody to CD4 led to rapid Fas-based apoptosis of T cells (92). Human T cell lines transformed with HIV were more sensitive to Fas-mediated apoptosis than the parental cells (15). These data are consistent with the involvement of Fas in the pathology of acquired immunodeficiency syndrome (AIDS), and this potentially important line of research is now being actively pursued in several laboratories.

An injection of monoclonal anti-Fas into adult mice caused rapid hepatic failure and death (83), suggesting that acute fulminant hepatitis in humans may be Fas-mediated. Accumulating data such as the involvement of specific CTLs in fulminant hepatitis (93), the sensitivity of primary hepatocytes to Fas-mediated apoptosis in vitro (94), and the overexpression of Fas in hepatocytes transformed with human hepatitis C virus (HCV) (95) are consistent with this hypothesis. In this model, virus antigens of hepatitis B virus or HCV expressed on hepatocytes would activate CTLs to express FasL, which then would bind to Fas on hepatocytes, inducing them to undergo apoptosis. This process may normally occur to remove virus-infected cells but, if exaggerated, may lead to fulminant hepatitis.

Fas is abundantly expressed not only in the liver but also in the heart and lungs (9). The primary cells from these tissues are sensitive to Fas-mediated apoptosis (96), suggesting that the Fas system may also be involved in CTL-mediated diseases in these tissues. The lymphocytes in *lpr* mice constitutively express FasL (69), which may be responsible for the graft-versus-host disease (GVHD) observed when *lpr* bone marrow is transferred to wild-type mice (97). These

results suggest that the Fas system may also be involved in GVHD induced by allogenic bone marrow transplantations in humans. Furthermore, a functional soluble form of the FasL was recently identified in the culture medium of activated human T cells (29). If such a molecule is produced in vivo, it may work as a pathological agent to cause systemic tissue injury.

Therapeutic uses of the Fas system might include blocking the exacerbated Fas-based pathological manifestations with either soluble forms of Fas, neutralizing antibodies to Fas or FasL, or inhibitors of FasL induction or Fas-mediated apoptosis. Some groups considered using Fas-based reagents (such as activating anti-Fas or FasL) for human cancer patients (15, 98). To avoid the potential risks of these reagents for normal tissues (83), it would be necessary to find a way to accurately target the reagents to the tumor cells.

Conclusions

Cell proliferation, differentiation, and survival are often regulated by growth, differentiation, and survival factors, respectively, which are collectively called cytokines. Cytokines bind to their complementary receptors, which transduce the extracellular signal into an intracellular signaling cascade. As described above, characterization of Fas and FasL revealed that, in some cases, cytokines can induce cell death. FasL binds to its receptor and kills the cell within hours by inducing apoptosis. Whereas dozens of factors are known to promote growth, differentiation, or survival, only a few cytokines, including FasL and tumor necrosis factor (TNF), have been found to induce apoptosis. The Fas and TNF systems seem to be mainly restricted to the immune system. It is possible that there are other death-inducing molecules functionally related to FasL that may help remove unwanted cells in nonlymphoid tissues and organs.

Binding of a growth factor to its receptor usually induces dimerization of the receptor (homodimerization or heterodimerization). The dimerized receptor then activates a cascade of intracellular reactions, which eventually induce the gene expression necessary for cell proliferation or differentiation. On the other hand, binding of FasL to its receptor probably induces trimerization of the receptor, which can then transduce the death signal. The fact that Fas-mediated apoptosis can occur without a nucleus indicates that no specific gene induction is required for this death process. The cells seem to have an intrinsic death program in the cytoplasm, which Fas may activate. This death program may be identical to that involved in apoptosis induced in other ways, for example, by the deprivation of survival factors. Some cells are resistant to Fas-mediated apoptosis, suggesting that they either lack this death program or express molecules that inhibit the signals induced by Fas or the death program itself. Biochemical characterization of this death program and its eventual inhibitor proteins in the cytoplasm may reveal other signaling intermediates.

The identification of FasL as a cell death–inducing molecule suggested that the Fas-FasL system has a pathological role in humans. It is known that TNF works as a cachectin and mediates septic shock. Like TNF, FasL may work as an agent that causes tissue damage. The gain-of-function mutation of the growth factor system causes cellular transformation, whereas the loss-of-function mutation of the Fas system (*lpr* or *gld* mutation) causes lymphadenopathy. In this regard, Fas and FasL may be considered as tumor suppressor genes. It is possible that one or more mutations in oncogenes or tumor suppressor genes in addition to the Fas-FasL mutation causes cellular transformation. Further elucidation of the mechanism of Fas-mediated apoptosis and of its role in physiology or pathology should contribute to a better understanding of not only the life and death of cells but also of the basic mechanism of some human diseases.

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Apoptosis in the Pathogenesis and Treatment of Disease

Craig B. Thompson

In multicellular organisms, homeostasis is maintained through a balance between cell proliferation and cell death. Although much is known about the control of cell proliferation, less is known about the control of cell death. Physiologic cell death occurs primarily through an evolutionarily conserved form of cell suicide termed apoptosis. The decision of a cell to undergo apoptosis can be influenced by a wide variety of regulatory stimuli. Recent evidence suggests that alterations in cell survival contribute to the pathogenesis of a number of human diseases, including cancer, viral infections, autoimmune diseases, neurodegenerative disorders, and AIDS (acquired immunodeficiency syndrome). Treatments designed to specifically alter the apoptotic threshold may have the potential to change the natural progression of some of these diseases.

The survival of multicellular organisms depends on the function of a diverse set of differentiated cell types. Once development is complete, the viability of the organism depends on the maintenance and renewal of these diverse lineages. Within vertebrates, different cell types vary widely in the mechanisms by which they maintain themselves over the life of the organism. Blood cells, for instance, undergo constant renewal from hematopoietic progenitor cells. In addition, lymphocytes and cells within the reproductive organs undergo cyclical expansions and contractions as they participate in host defense and reproduction, respectively. In contrast, neural cells have at best a limited capacity for self-renewal, and most neurons survive for the life of the organism.

Within each lineage, the control of cell number is determined by a balance between cell proliferation and cell death (Fig. 1). Cell proliferation is a highly regulated process with numerous checks and balances. For example, growth factors and proto-oncogenes are positive regulators of cell cycle progression (*1*). In contrast, tumor suppressor genes act to oppose uncontrolled cell proliferation (*1*, *2*). Tumor suppressors can prevent cell cycle progression by inhibiting the activity of proto-oncogenes. In the last 15 years there has been a rapid increase in our understanding of the mechanisms that control cell proliferation.

Biologists are now beginning to appreciate that the regulation of cell death is just as complex as the regulation of cell proliferation (*3*). The differentiated cells of multicellular organisms all appear to share the ability to carry out their own death through activation of an internally encoded suicide program (*4*). When activated, this suicide program initiates a characteristic form of cell death called apoptosis (*5*, *6*). Apoptosis can be triggered by a variety of extrinsic and intrinsic signals (*7*) (Fig. 2). This type of regulation allows for the elimination of cells that have been produced in excess, that have developed improperly, or that have sustained genetic damage. Although diverse signals can induce apoptosis in a wide variety of cell types, a number of evolutionarily conserved genes regulate a final common cell death pathway that is conserved from worms to humans (*8*) (Fig. 3).

Apoptotic cell death can be distinguished from necrotic cell death (*4*–*6*). Necrotic cell death is a pathologic form of cell death resulting from acute cellular injury, which is typified by rapid cell swelling and lysis. In contrast, apoptotic cell death is

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